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GENOMIC INSTABILITY AND LIPID PEROXIDATION IN PATIENTS WITH TREATED ESSENTIAL HYPERTENSION

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ABSTRACT

Impaired oxidant/antioxidant status in hypertension results in increased oxidative stress. This may cause genomic instability, damage membrane proteins and lipid peroxidation in hypertensive individuals. Aim: The aim of this study was to assess genomic instability (DNA damage) and lipid peroxidation in treated hypertensive patients belonging to a population sub-group. Materials and Methods: A case-control study was carried out on amlodipine-atenolol treated hypertensive patients (n=22) and normotensive (n=10) control group, belonging to same sub-group. The study was approved by Institutional Ethics Committee. The demographic, physiometric, anthropometric variables were recoded after the written informed consent. The leukocyte DNA damage by the single cell gel electrophoresis assay and malondialdehyde (MDA) levels using standard protocols were assessed in the study group to investigate genetic damage and oxidative stress. The data was expressed as mean ± S.E.M. Chi-square, student's t-test, ANOVA, Pearson correlation and multiple linear regression were used to compare and find association if any of the damage parameter with the confounding variables. Results: DNA damage was significantly elevated (p=0.003) in the hypertensive patients compared to value in normotensive individuals. MDA levels were also significantly increased in patients. Conclusion: The increased genetic damage and levels of lipid peroxidation in hypertensive patients were associated with the elevated blood pressure, abnormal lipid profile, obesity and drug treatment. The genomic instability probably resulted as a consequence of oxidative stress from these variables and drug treatment increase the risk for cardiovascular diseases, target-organ damage and carcinogenesis.

Keywords: DNA damage, malondialdehyde, lipid profile, blood pressure, carcinogenesis.

1. INTRODUCTION

The association between elevated blood pressure and cancer risk has been a matter of concern with reports available on increased incidence of kidney, liver, endometrium and pancreatic cancer (Zhang *et al.*1996; Chow *et al*. 2000; Furberg and Thune, 2003). Increased oxidative stress (cause-effect relationship) in hypertension(de Champlain et al. 2004; Yildiz *et al*. 2008) as well as antihypertensive therapy (Chin *et al*.2003), higher body mass index (Brown *et al*. 2009), have been reported to increase cancer incidence in hypertensive patients. Among these though the mode of action of commonly prescribed antihypertensive drugs on cancer is unknown. The other factors (physiological stress, BMI as well as smoking and alcohol) have a significant relation with overproduction of reactive oxygen species/reactive nitrogen species (ROS/RNS) (Khanna *et al*. 2008). The impaired oxidant/antioxidant balance in hypertension causes increased levels of reactive oxygen species, that can

target the cellular molecules and cause their oxidation (Briones and Touyz, 2009) Lipid peroxidation, oxidized C-reactive proteins, oxidative DNA damage as well as other mutational events resulting from genetic damage, can trigger the carcinogenesis process (Marnebtt, 2000 ; Thompson, 2000). In the present investigation it was therefore pertinentto determine if treated hypertensive patients have the potential to accumulate increased levels of lipid peroxidation and DNA damage. Only a few reports on lipid peroxidation levels (Mahajan *et al*. 2007) and DNA damage in hypertensive patients exist while the study on patients from this region are sparse (Gandhi and Jyoti, 2010) and none on those taking the amlodipine-atenolol combination. As blood pressure is the pressure of the blood within the arteries blood produced by the contraction of the heart muscles the appropriateness of assessing peripheral blood leukocytes for DNA damage as a pre-clinical carcinogenic lesion gains importance; lipid peroxidation was estimated in serum samples. The alkaline single cell gel electrophoresis (SCGE/comet) assay is a sensitive technique for assessing genomic (DNA) damage (Singh *et al*. 1988) as it can detect single-strand and double-strand DNA breaks and alkali-labile sites which manifest as a tail from the nucleoid under electrophoresis giving, the appearance of a comet; the tail length and intensity indicate the amount of DNA damage. The assay has been used to assess DNA damage in patients of diabetes (Blasiak *et al*. 2004), coronary artery disease (Demirbag *et al*. 2005) and hypertension (Gur *et al*. 2007).The peripheral blood leukocytes of hypertensive patients on amlodipine-atenolol combinational therapy were assessed for DNA damage. Lipid peroxidation was measured by spectrophotometric analysis of malondialdehyde (MDA) (Beuge and Aust, 1978).Normotensive healthy individuals matched for age, sex and other lifestyle variables, comprised the control group.

2. MATERIAL AND METHODS

2.1. Subjects: A case-control study (n=32) was carried out on Amlomed AT containing Amlodipine($C_{20}H_{25}ClN_2O_5$)-5mg and Atenolol ($C_{14}H_{22}N_{12}O_3$)-50mg- treated hypertensive patients (n=22) and on healthy normotensive subjects (n=10). The patients were enrolled from the Guru Ram Das Charitable Hospital, Amritsar and belonged to the Arora Sikh population sub-group. Age- and sex-matched healthy normotensive individuals belonging to the same sub-group from the general population comprised the control group. The study was approved by the Institutional Ethics Committee. After the participants gave voluntary written informed consent, on a pre-designed questionnaire, their personal demographic and disease details were recorded. Blood Pressure measurements from the right upper arm were taken for each participant in the sitting position using a mercury sphygmomanometer. The average of three readings taken at ten min. intervals was recorded as the blood pressure readings. The anthropometric variables (height and weight) were taken as per standard procedures (Weiner and Laurie, 1981) to calculate the body mass index (BMI, kg/m^2) and the waist-hip ratio (WHR) and obesity cut-off were as given by WHO, respectively (Wolf and Smith, 2005; Misra *et al*. 2009).

2.2. Sample collection: Peripheral venous blood samples (~5ml) were drawn from each subject and placed into heparinized and non-heparinized vials for DNA damage and malondialdehyde (MDA) assessment, respectively. The SCGE assay was performed on leukocytes and the lipid profile and MDA analysis was carried out on blood-sera samples. Locally available chemicals were used.

2.3. Biochemical Analysis: Serum was prepared by allowing the blood to clot by leaving it undisturbed at room temperature and then centrifugation was carried out to remove clot at 1,000-2,000 x g for 10 minutes in a centrifuge. Serum levels of triglycerides (TG), total cholesterol (TC) and of high density lipoprotein-cholesterol (HDL-C) were determined on an semi-automated analyzer (ERBA-CHEM 7) using commercial kits (Angstorm, INDIA). The low density lipoproteins-cholesterol (LDL-C) was calculated using Friedwald's formula.

2.4. Malondialdehyde Measurement: MDA levels were determined by the colorimetric method as per Beuge and Aust (1978). The serum sample (0.1 ml)

was mixed thoroughly with 0.1 ml of Tris HCl and then 0.1 ml of ascorbic acid and 0.1 ml of ferrous ammonium sulfate were added. The final volume was made upto one ml by adding double-distilled water and it was incubated for 15 min at 37°C. For coupling of lipoproteins thus precipitated and thiobarbituric acid (TBA), 1 ml of trichloroacetic acid and 2 ml of TBA were added and placed in a water bath (~100°C) for 15 min followed by centrifugation at 1000 rpm for 15 min. The absorbance of the resulting chromogen was then determined at 532 nm against the blank (all reagents and normal saline instead of serum sample). The concentration of MDA was then calculated Jyothi *et al*. (2008).

2.5. The Single Cell Gel Electrophoresis Assay: Leukocyte DNA damage was analyzed by the alkaline SCGE/Comet assay as described [15] with minor changes. Freshly heparinized blood (30 µl) was mixed with 100 µl of 0.5% low melting point agarose (LMPA) in PBS at 37°C and 100 µl of it was sandwiched between two layers of normal melting point agarose (1% NMPA) and LMPA on a clean slide. Two slides were prepared per sample. The slides were covered with a coverslip and kept at 4°C for 15 min to allow the agarose to solidify. This was followed by lysing in freshly prepared cold lysing solution for 2-3 h. The slides were then immersed in freshly prepared alkaline electrophoresis buffer at 4°C for unwinding (30 min) and then electrophoresed (25V; 300mA, 25 min). After neutralization (10 min), slide preparations were stained with silver nitrate (Garcia *et al*. 2006) and analyzed using an image analysis software programme (Comet Assay Software Program; CASP, http://casplab.com/) to find tail DNA percent. The percentage of tail in DNA reflects the proportion of DNA that has migrated from the head and it is the most informative of SCGE assay parameters.

2.6. Statistical Analysis: Results are presented as mean± S.E.M. Demographic variables were compared using the Chi-square test. Differences between control and hypertensive subjects were assessed using the Student`s t-test as data were parametric. Associations between DNA damage, MDA, BP and other variables were evaluated by Pearson`s correlation test. The independent association of these variables and confounding factors was evaluated by the multiple linear regression analysis. The comparisons between groups were performed by one-way analysis of variance (ANOVA). Values less than p= 0.05 were considered statistically significant.

3. RESULTS

DNA damage in PBL was significantly elevated (p=0.003) in the hypertensive patients compared to value in normotensive individuals. Lipid peroxidation (MDA) was also significantly increased in patients (p=0.000). Table 1 depicts the demographic and clinical characteristics of patients and controls. The study group comprised treated hypertensive patients (males n=11; average age 68.27±1.17y; and blood pressure 156.36/93.18 mmHg) and females (n=11, average age 66.28±0.94y and blood pressure 151.82/88.636 mmHg). All the patients were on combinational drug therapy (Amlomed AT; 55mg/day) for 1-3 years. The control group comprised normal, healthy individuals (males n=6; females n=4) matched for age, sex, diet, etc. but differing in clinical parameters. On applying the Student`s t-test on patients and controls (Table 2) for baseline characteristics, BMI, blood pressure measurements and lipid profile (except HDL), MDA and genetic damage (percent tail DNA) were significantly elevated in the patient group (Table 3). Within the patient and control groups no gender differences were observed for these characteristics. In male group (patient vs. control), no significance was observed for BMI and WHR; similarly in the female group, HDL also showed no significance. A significant positive correlation was however also observed of DNA damage with age, BMI, WHR, systolic blood pressure (SBP), diet, mobile usage, TC, TG, LDL-C, pulse pressure (PP) and mean arterial pressure (MAP).Multivariate ANOVA revealed the significant association of various confounding demographic, clinical and biochemical parameters except DBP, alcohol, and triglycerides in the patients. Linear regression analysis further revealed that DNA damage was influenced by age,

BMI, WHR,SBP, PP, MAP, diet, mobile phone usage and most of the lipid profile markers.

Table 1.*Demographic and clinical characteristics of treated hypertensive patients and controls*

Parameter	Range	Patients(n=22)	Controls(n=10)	χ^2
Age(y)	40-64	13	5	0.231
	65-84	9	5	
Sex	male	11	6	0.083
	female	11	4	
Height(cm)	144-166	9	5	0.0231
	167-186	13	5	
Weight(Kg)	50-73	12	6	0.083
	74-96	10	4	
Diet	Veg/Non Veg	14/8	8/2	0.186
Alcohol drinking	Yes/No	7/15	2/8	0.475
Smoking	Yes/No	2/20	0/10	0.965
SBP(mmHg)†	>120-139	-	10	**47.6**
	140-159	16	-	
	160-179	4	-	
	180-200	2	-	
DBP(mmHg)†	70-89	18	10	**50.02**
	90-100	3	-	
	101-111	1	-	
PP(mmHg)†	40-70	7	10	**28.72**
	70-100	15	-	
MAP(mmHg)†	90-110	8	10	**40.72**
	111-130	14	-	

Values in bold are significant (χ^2-test,p<0.05)
(SBP-Systolic Blood Pressure ,DBP-Diastolic Blood Pressure,PP-Pulse Pressure MAP-Mean Arterial Pressure)
†-As per JNC VII

Table 2: ***The comparison of baseline characteristics of the patient and control group***

CHARACTERISTICS	VARIABLES	PATIENT GROUP (n=22)			CONTROL GROUP (n=10)		
		Males(n=11)	Females(n=11)	Total(n=22)	Males(n=6)	Females(n=4)	Total(n=10)
Obesity status	BMI(kg/m^2)	26.169±1.305	28.097±1.831	27.133±1.174	24.838±1.196	22.431±0.546	23.875±0.819
	WHR	1.033±0.028	1.023±0.022	1.028±0.0172	1.0317±0.008	0.971±0.013	1.007±0.122

Physiometric measurements	SBP(mmHg)	156.36*±6.609	151.82*±5.405	154.09**±4.195	131.67±2.789	123.75±2.394	128.50±2.242
	DBP(mmHg)	93.181**±2.719	88.636**±2.344	90.909**±1.821	76.67±2.472	77.50±4.33	77.00±2.134
	PP(mmHg)	63.182*±5.927	63.181*±6.917	63.182*±4.445	56.00±3.416	47.50±5.951	52.00±3.198
	MAP(mmHg)	114.03**±2.965	109.49*±2.357	111.76**±1.914	94.817±2.02	93.175±3.603	94.160±1.779
Lipid profile	TC (mg/dl)	236.47***±17.131	205.25**±15.173	301.91**±9.012	185.53±17.391	168.20±23.174	178.60±13.430
	LDL-C (mg/dL)	228.13***±12.008	206.79**±13.837	164.43**±9.709	179.92±15.736	154.68±5.588	99.498±13.008
	HDL-C (mg/dL)	38.99±12.932	32.152±3.218	35.095±2.549	39.90±5.096	40.535±2.563	40.155±3.089
	TG (mg/dL)	152.81**±18.582	131.74*±14.303	209.60*±10.778	104.66±16.954	91.750±22.737	169.82±10.183
Lipid peroxidation	MDA (µmol/l)	2.056**±0.155	1.963**±0.101	2.009***±0.091	1.057±0.191	1.00±0.319	1.034± 0.161
DNA damage	Tail DNA Percent	10.952*±1.110	10.682*±0.944	10.817**±0.712	8.038±0.856	6.367±1.487	7.369±0.783

Values are significant at p≤0.001(***),p≤0.01(**),p≤0.05(*) Vs Control group (Student's t-test)
(SBP-Systolic Blood Pressure ,DBP-Diastolic Blood Pressure,PP-Pulse Pressure, MAP-Mean Arterial Pressure,TC-Total Cholesterol, LDL-Low Density Lipoproteins,HDL-High Density Lipoproteins,TG-Tri glycerides, MDA-Malondialdehyde)

Table 3:*Multivariate ANOVA, Pearson Correlation and Multiple Linear Regression analysis of tail DNA % and variables*

VARIABLES	PATIENT GROUP(n=22)				CONTROL GROUP(n=10)			
	Multivariate ANOVA(F)	Pearson correlation (r)	Regression coefficient (95%CI)	p-value	Multivariate ANOVA(F)	Pearson correlation(r)	Regression coefficient $_{\beta}$(95%CI)	p-value
Age(yrs.)	35.541	0.800	o.800 (3.392-7.043)	**0.000**	2.604	-0.496	-0.496 (-0.245-0.043)	0.145
BMI(kg/m^2)	8.309	0.542	0.542 (0.095-0.595)	**0.009**	2.079	-0.454	-0.454 (-5.549-1.280)	0.187
WHR	8.422	0.544	0.544 (6.320-38.626)	**0.009**	1.202	0.361	0.361 (-0.381-1.073)	0.305
SBP(mmHg)	38.582	0.812	0.812 (0.091-0.184)	**0.000**	0.661	0.276	0.276 (-32.618-68.152)	0.440
DBP (mmHg)	0.062	0.056	0.056 (-0.160-0.204)	0.805	0.000	0.001	0.001 (-0.284-0.285)	0.998
PP((mmHg)	24.669	0.743	0.743 (0.069-0.169)	**0.000**	1.057	0.342	0.342(-0.105-0.273	0.334
MAP(mmHg)	12.669	0.623	0.624 (0.096-0.367)	**0.002**	0.133	0.128	0.128(-0.300-0.413	0.725
TC(mg/dl)	19.336	0.701	0.701	**0.000**	0.333	0.200	0.200	0.580

			(0.022-0.063)				(-0.030-0.058)	
HDL(mg/dl)	0.577	-0.167	-0.167 (-0.175-0.082)	0.456	0.107	0.115	0.115(-0.176-0.235)	0.752
LDL(mg/dl)	23.205	0.733	0.733(0.025-0.064)	**0.000**	0.421	0.224	0.224 (-0.034-0.061)	0.535
TG(mg/dl)	0.006	0.017	0.017(-0.035-0.037)	0.940	0.397	0.217	0.217(-0.044-0.078)	0.546
Diet	4.889	0.443	0.443 (0.170-5.839)	**0.039**	0.030	-0.062	-0.062 (-6.389-2.709)	0.866
Alcohol drinking	2.538	-0.336	-0.336	0.127	0.870	-0.313	0.313 (-2.709-6.389)	0.378
Mobile usage	8.120	0.537	0.537 (1.121-7.243)	**0.010**	-	-	-	-

Values in bold are significant

Table 4: ***Multivariate ANOVA, Pearson Correlation and Multiple Linear Regression analysis of Percent tail DNA and MDA according to gender***

		PATIENT GROUP (n=22)			CONTROL GROUP (n=10)		
		Males(n=11)	Females(n=11)	Total(n=22)	Males(n=6)	Females(n=4)	Total(n=10)
Tail DNA %		10.952±1.110	10.682±0.944	10.817±0.712	8.038±0.856	6.367±1.487	7.369±0.783
MDA (µmol/l)		2.056±0.155	1.963±0.101	2.009±0.091	1.057±0.191	1.00±0.319	1.034±0.161
ANOVA(F)		58.749	2.034	25.042	13.202	22.938	27.859
Regression	Beta	0.931	0.429	0.746	0.876	0.959	0.881
	CI(95%)	(4.707-8.649)	(-2.355-10.391)	(3.411-8.287)	(0.925-6.919)	(0.454-8.478)	(2.417-6.168)
	t	3.633	4.789	5.278	7.665	1.426	5.004
Pearson correlation(r)		0.876	0.959	0.881	0.931	0.429	0.746
p-value		**0.022**	**0.041**	**0.001**	**0.000**	0.188	**0.000**

Values in bold are significant
CI-Confidence Interval

In Table 4 are presented the genetic damage and oxidative stress indices according to gender. Genetic damage was very slightly higher in male patients compared to female patients as were the MDA levels. However these values were significant (p=0.002) in comparison to sex-matched control groups. Since oxidative stress can cause DNA damage, the relationship of MDA levels with the corresponding DNA damage in male and female patients was ascertained. Association was observed only in male patients (p=0.000) and in the total patient group (p=0.000). A positive correlation was however observed in all groups indicating that increase in MDA levels could correspondingly affect the induced genetic damage.

4. DISCUSSIONS

In the present study, leukocyte DNA damage was increased (p=0.003) in treated hypertensive patients compared to normotensive control individuals; no gender differences were however observed. Oxidative stress (MDA) was also significantly

elevated (p=0.000) in patients as were the lipid biomarkers (total cholesterol, p=0.002; LDL-C, p=0.001; triglycerides, p=0.012). The patients were on a single treatment regimen for 1-3 years comprising a daily dose of a single combinational drug (Atenolol+amlodipine). DNA damage was significantly associated with age, BMI, WHR, SBP, PP, MAP, TC, LDL-C, diet and mobile usage. These results find consistence with various earlier studies.

Essential hypertensive north Indian patients had significant genetic damage as observed in the SCGE assay and a correlation with BMI and BP was also observed (Khanna *et al*.2008). The increased lymphocyte DNA damage was observed in patients with white coat hypertension which was attributed to a decrease in total antioxidant status (Yildiz *et al*. 2008). Decrease in total antioxidant status can result from elevated oxidative stress which has also been widely reported(Briones and Touyz, 2009). In another study it was demonstrated that DNA damage in hypertensive patients was repaired by additional antioxidant intake (grape juice) (Park *et al*. 2009). In a Turkish population with coronary artery disease (CAD), increased genetic damage was reported which was attributed to severity of CAD and low levels of total antioxidants (Gur *et al*. 2007).

The state of hypertension is associated with oxidative stress due to impaired oxidant/antioxidant status. The oxidative stress in hypertension arises when ROS exceeds the level of antioxidant defense system (Yusuf *et al*. 2004). Rather a constant increase in ROS increases blood pressure and vice-versa (Ward *et al*. 2004). Endothelial dysfunction causes defect in the vasodilator which inactivate nitric oxide (NO) and causing oxidative stress (Gandhi and Jyoti 2010). Increased xanthine oxidase activity has also been observed in hypertension (Marnebtt 2000). The decreases activity of superoxide dismutase and catalase and reduced levels of ROS scavengers (vitamin E, Glutathione) also may be contributors of oxidative stress in hypertension (Alghasham *et al*. 2011). In this study, the increase in oxidative stress as elevated MDA levels (p=0.000) was found in treated hypertensive patients indicates increased oxidative stress in them. The positive correlation was also found between DNA damage and MDA levels suggesting that increased DNA damage may be due to increased lipid peroxidation in hypertension. A positive correlation between MDA levels and elevated blood pressure in hypertensive patients was found (Sahu *et al*. 2009). Similar results were observed in pregnancy-induced hypertension (Niedernhofer *et al*. 2003).

Compared to controls, the elevated levels of lipids and MDA in the patients of the present study suggest that oxidative stress may be one of the causes for genetic damage in hypertensive patients. MDA forms adducts with DNA which are pro-mutagenic and can lead to target organ damage and cancer (Telez *et al*. 2000). As MDA is a biomarker of lipid peroxidation an increase in lipid profile markers as observed in the patients of this study for more lipid oxidation products.

Essential hypertension requires regular long-term anti-hypertensive treatment to maintain the levels of blood pressure. The patients in the present study were on amlodipine-atenolol combined therapy. Amlodipine acts by relaxing the smooth muscles in arterial wall, decreasing peripheral resistance, and hence, reducing blood pressure, on the other hand, atenolol is a β-blocker, which exerts a greater blocking activity on myocardial β-1-receptors, leading to inhibition of secretion of renin and decreasing heart rate and contractibility. Atenolol induced a significant increase in micronuclei in treated hypertensive patients (Robbiano *et al*.1992); through sister chromatid exchanges were not increased. In fact the drug caused aneuploidy in cultured lymphocytes as revealed by the presence of centromere- positive micronuclei. The genotoxicity of atenolol in human hepatocytes was also reported (Télez *et al*. 2010). Recently, higher incidence of chromosomal aberrations and more expression of fragile site in patients on atenolol-therapy were observed while fluorescent in-situ hybridization analysis revealed the preferential involvement of chromosomes 7 and 11 in the aneugenic-potential of atenolol. The reports on effects of amlodipine have documented no effects on bacterial mutagenecity or on chromosomal aberrations in mice while lipid peroxidation was significantly inhibited by it (Ahr *et al*. 1997; Harrison *et al*. 2007). Nuclear changes associated with apoptosis at nanomolar levels were also inhibited a neuro-protective activity was induced and

there was decreased formation of ROS by aneuploidy (Mason *et al*. 1999).

Study limitations: The present study has a small sample size; a larger sample size can yield more meaningful results. In this study single genetic damage and oxidative stress parameters have been evaluated. Other oxidative stress and genetic damage indices need to be investigated for an exact oxidative stress status and genomic instability.

5. CONCLUSION

The study highlights for the first time significantly increased genetic damage and lipid peroxidation levels in peripheral blood leukocytes of some North Indian hypertensive Arora sikh patients on atenolol-amlodipine combination-therapy. The significantly increased DNA damage requires close monitoring, since events in carcinogenesis and the development of degenerative and mitochondrial diseases get initiated from such DNA lesions.

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